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High *Wolbachia* Strain Diversity in a Clade of Dung Beetles Endemic to Madagascar

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Determining the drivers of diversity is a major topic in biology. Due to its high level of micro-endemism in many taxa, Madagascar has been described as one of Earth's biodiversity hotspot. The exceptional Malagasy biodiversity has been shown to be the result of various eco-evolutionary mechanisms that have taken place on this large island since its isolation from other landmasses. Extensive phylogenetic analyses have, for example, revealed that most of the dung beetle radiation events have arisen due to allopatric speciation, and adaptation to altitudinal and/or longitudinal gradients. But other biotic factors, that have yet to be identified, might also be at play. *Wolbachia* is a maternally transmitted endosymbiotic bacterium widespread in insects. The bacterium is well-known for its ability to modify its host reproductive system in ways that may lead to either discordance patterns between the host mitochondrial and nuclear phylogenies, and in some cases to speciation. Here, we used the MultiLocus Sequence Typing system, to identify and characterize five *Wolbachia* strains infecting several species within the *Nanos clypeatus* dung beetle clade. We discuss the implications of these *Wolbachia* strains for the evolution and diversification of their dung beetle hosts in Madagascar.

Keywords: endosymbiont, speciation, endemism, phylogeny, introgression

INTRODUCTION

With roughly 200,000 species of which at least 90% are endemic to the Island, Madagascar is one of the Earth's richest biodiversity hotspots (Paulian, 1987; Myers et al., 2000; Vences et al., 2009). For Malagasy dung beetles, the level of endemism reaches 96% (Miraldo et al., 2011). Like for many other organisms that have evolved on the island since its isolation from other land masses since the late Cretaceous era (Noonan and Chippindale, 2006), the high level of species radiation in dung beetles has been attributed to different eco-evolutionary mechanisms. Strong environmental gradients and ecosystem heterogeneity across the island have often been suggested as some of the main drivers of the Island's exceptional biodiversity (Vences et al., 2009; Miraldo and Hanski, 2014). In contrast, little is known about the potential influence of biotic factors, such as the presence of endosymbionts, in the diversification of Malagasy species.

The genus *Nanos* includes 42 species of dung beetles endemic to Madagascar (Montreuil et al., 2014). This clade is considered one of the most recent and ecologically successful dung beetle lineages in Madagascar (Wirta, 2009; Wirta et al., 2010). The phylogeny of this clade was recently revisited by combining morphological and molecular data from both mitochondrial and

nuclear markers (Wirta, 2009; Montreuil et al., 2014). This work improved our understanding of the phylogenetic relationship between many morphologically similar *Nanos* species, but also highlighted potential introgression between species, especially within the *N. clypeatus* group (Wirta et al., 2008; Wirta, 2009; Montreuil et al., 2014). Furthermore, Wirta (2009) showed that the mitochondrial DNA of the six species from the *N. clypeatus* group was less variable than that of the rest of the *Nanos* species.

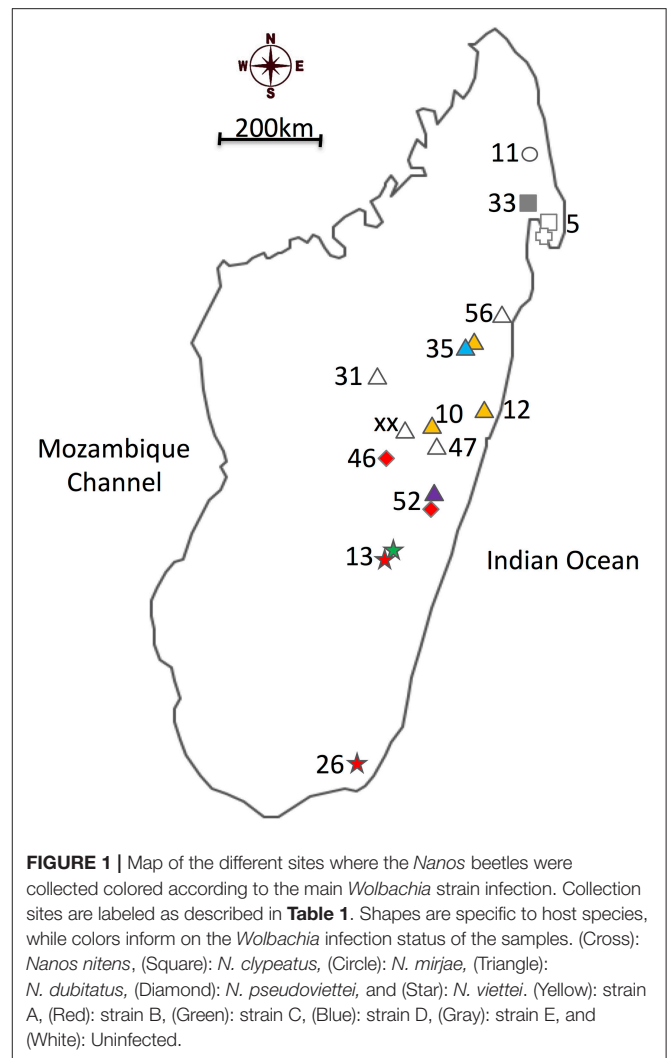
Maternally inherited endosymbiotic bacteria act as a selfish entities that can modify their host reproductive strategies to their own benefit (O'Neill et al., 1997; Dyson and Hurst, 2004; Engelstädter and Hurst, 2007). Text-book examples include the association between *Wolbachia* strains inducing cytoplasmic incompatibility (CI) in their hosts. Due to CI, the zygotes of uninfected female hosts mated to *Wolbachia*-infected males die, while zygotes from *Wolbachia*-infected females thrive in the environment (Carrington et al., 2011; Kriesner et al., 2013; Turelli et al., 2018). In these conditions, *Wolbachia* can obscure studies of mitochondrial variation in the host population. The mitochondrial selective sweep associated with the invasion of the inherited endosymbiotic bacteria can indeed reduce the mitochondrial diversity observed in infected lineages when compared to uninfected ones, and confuse conclusions from phylogenetic studies (Jiggins, 2003; Charlat et al., 2009; Jäckel et al., 2013; Kriesner et al., 2013; Mazur et al., 2016; Cariou et al., 2017). Furthermore, studies have suggested that the induction of CI can also select for premating isolation between matriline carrying different infection status (Telschow et al., 2005a, 2007). The selection pressure for the evolution of mate-choice strategies toward individuals carrying the same infection status can lead to strong premating isolation, which in time will support reproductive isolation between the host lineages, and lead to speciation (Telschow et al., 2005a, 2007; Cariou et al., 2017).

Here we suggest that infections with *Wolbachia* strains might play a role in the loss of mitochondrial diversity observed in the *N. clypeatus* group, which includes six dung beetle species. We also hypothesize that incompatible *Wolbachia* infections could keep the different taxa as clear evolutionary units despite clear genetic introgression patterns between the *N. dubitatus* and *N. vieittei* species. We discuss the potential implications of these findings for the evolution and diversity of the dung beetles in Madagascar.

MATERIALS AND METHODS

Material

The *Nanos* beetles used in this study were collected across Madagascar (Figure 1) between 2002 and 2008, for the purpose of several other studies investigating speciation in the Malagasy *Nanos* clade (Wirta, 2009; Miraldo and Hanski, 2014). Beetles were collected with standard baited pitfall-traps. The 1,5 dl plastic cups were filled to one third with water and dishwashing liquid to decrease water tension upon contact with beetles. A bait of raw fish ($V = 3 \text{ cm}^3$) was hanged over each cup to attract the beetles. Each trap was covered by a large leave or a plastic plate to prevent overflow with rain water. Duploux



et al. (2009) previously showed that *Wolbachia* cross-specimens contamination was not occurring during collection and storage in similar conditions.

Our study focuses on the six species composing the “*Nanos clypeatus* clade,” namely *N. clypeatus*, *N. dubitatus*, *N. vieittei*, *N. pseudoviettei*, *N. mirjae*, and *N. nitens* (Miraldo and Hanski, 2014; Montreuil et al., 2014). The species have differentiated at most 7 Mya, and show very little geographical overlap across Madagascar, due to strong niche competition between the species. The three most common species, *N. vieittei*, *N. dubitatus*, and *N. clypeatus*, are, respectively, found in the South Eastern region, the Northern half region, and around the Masoala peninsula region of Madagascar (Wirta, 2009). One exception occurs in central Madagascar, where *N. dubitatus*, and *N. pseudoviettei* occur in sympatry in the locality of Vohitrombo-Marolambo. It is at this particular locality that specimens showing patterns of introgression between the two species were previously collected (Viljanen, 2009a; Wirta, 2009; Viljanen et al., 2010). Table 1 and Figure 1 provides sample size for each species and population sampled.

TABLE 1 | Species, sample size, and *Wolbachia* infection status at each collection site.

Species	Collection site	Site ID	<i>Wolbachia</i> infection rate (N=)	<i>Wolbachia</i>	Haplotype count
<i>Nanos clypeatus</i>	Masoala-Andranobe	5	0% (0/7)	–	14
	Makira	33	11% (1/9)	wNcly_A	
<i>Nanos dubitatus</i>	Ambila	12	75% (3/4)	wNdub_A	17
	Ambohitantely	31	0% (0/5)	–	
	Andasibe	10	83% (5/6)	wNdub_A	
	Manasema-Lakato	47	0% (0/9)	–	
	Mandraka	XX	0% (0/1)	–	
	Tampolo	56	0% (0/8)	–	
	Vohitromby-Marolambo	52	43% (3/7)	UnSt	
	Zahamena	35	100% (6/6)	1xwNdub_A 1xwNdub2_A 4x multiply	
<i>Nanos mirjae</i>	Marojejy	11	0% (0/10)	–	6
<i>Nanos nitens</i>	Masoala-Andranobe	5	0% (0/9)	–	4
<i>Nanos veittei</i>	Andohahela	26	100% (10/10)	wNvie_A	12
	Ranomafana	13	100% (9/9)	7x wNvie_A 2x wNvie2_A	
<i>Nanos pseudoveittei</i>	Tsinjoarivo	46	100% (9/9)	wNvie_A	14
	Vohitromby-Marolambo	52	100% (10/10)	wNvie_A	
All species	Fourteen locations	NA	47% (56/119)	Five strain variants + 1x UnSt	67 mitotypes

UnSt, uncharacterized strain. The bold data in the site ID column represent sites where two beetle species co-occur.

Molecular Work

We individually extracted the DNA from 119 dung beetles using a Qiagen DNAeasy blood and tissues extraction kit and following the manufacturer's protocol (Qiagen, Germany). The *COI* mitochondrial gene was amplified by PCR using the primer pairs LCO/HCO (Folmer et al., 1994). Although endosymbiotic bacteria are taxonomically diverse; including members of the bacterial genera *Arsenophonus*, *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*; we here focused solely on infection by *Wolbachia*, which is considered the most common of them all, including in Coleopteran hosts (Kajtoch and Kotaskova, 2018; Kolasa et al., 2018). We screened for *Wolbachia* infection using five *Wolbachia* MLST markers (MultiLocus Sequence Typing genes: *coxA*, *fbpA*, *ftsZ*, *gatB*, and *hcpA*, using respective degenerate primers designed by Baldo et al. (2006). Amplicons from both reverse and forward strands were sequenced on an automated AB1-3730-DNA Analyser (Applied Biosystems™, USA). Sequences were checked for consistency between strands, and manually curated using Geneious R6 (<http://www.geneious.com>) (Kearse et al., 2012). All *Wolbachia* sequences were deposited to GenBank (GenBank # MK636654–MK636666). Unfortunately, as is often the case in many studies (Rodríguez et al., 2010; Roehrdanz and Wichmann, 2014), we failed to amplify each of the five MLST loci for several strains, and only assigned a full strain variant name when two or more of the MLST loci were sequenced. Variant names were assigned following the standard rules given on the pubMLST database (<http://pubmlst.org/wolbachia>) (Baldo et al., 2006). All sequences were compared to the pubMLST database using BLASTn (Baldo et al., 2006).

Phylogenetics

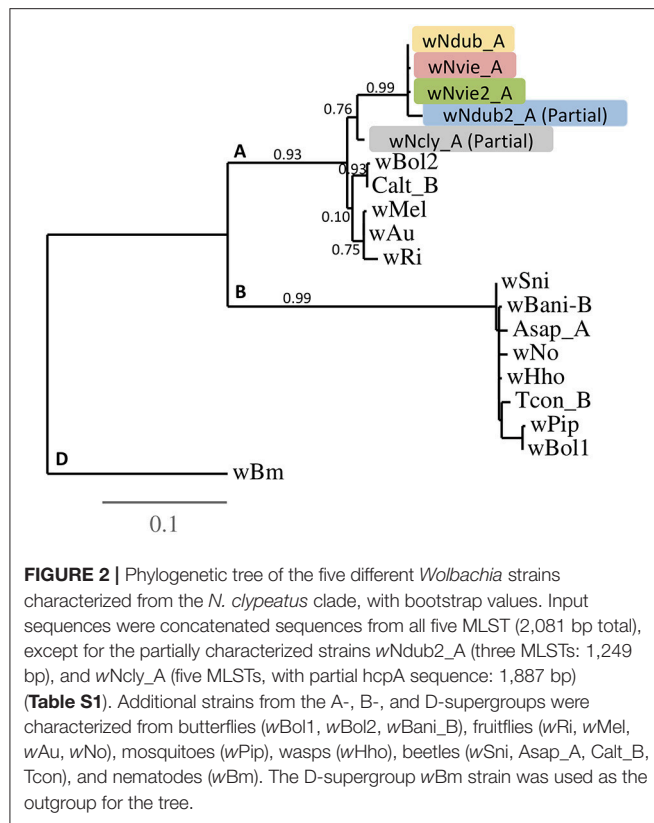
Phylogenetic trees were constructed using the online tree-building software *Phylogeny.fr* using the One Click mode with

default settings (Dereeper et al., 2008). In brief, the *One-Click* method builds a maximum likelihood tree using PhyML (Guindon et al., 2010) with sequence alignment using MUSCLE (Edgar, 2004a,b). We rooted the *COI* gene tree using the *COI* sequences from *N. manomboensis* and *N. binotatus* (GenBank KF309798 and KF309750) (Miraldo et al., 2011). According to Montreuil et al. (2014), *N. manobonensis* and *N. binotatus* belong to the *N. bimaculatus* group, which falls outside the “*N. clypeatus* clade.” The respective MLST sequences from 14 additional *Wolbachia* strains (wPip, wRi, wMel, wBm, wBol2, wBol1, wBani_B, and wHho; GenBank #AM999887, CP001391, AE017196, AE017321, EF025179–183, EF078895, AB474245–249, AB094382, KY658553, KY658572, KY658591, KY658610, KY658630, KF722987–992; and wAu, wNo, wSni, wAsap_A, wCalt_B, wTcon_B; Wolbachia-PubMLST id#10, #27, #301, #3, #19 and #20, respectively), previously characterized as belonging to the A-, B-, or D-supergroup, were added to the *Wolbachia* phylogenetic trees. We first constructed a phylogenetic tree for each MLST locus separately (Figure S1), and then built a phylogeny based on the concatenated sequences (Figure 2). We rooted each tree using the D-supergroup strain, wBm, as the outgroup.

RESULTS

Wolbachia Infections

We characterized a total of five *Wolbachia* strains (Figure 2) from four of the six species screened (*N. clypeatus*, *N. veittei*, *N. pseudoveittei*, and *N. dubitatus*). All strain variants belong to the A-supergroup, and show little divergence between each other. The wNdub_A and wNvie_A strain variants differ by one substitution out of 2081bp sequenced (99.95% similarity), wNdub_A and wNvie2_A also differ by one substitution at a



different position, while wNvie_A and wNvie2_A differ by two substitutions. Strains wNdub2_A and wNcly_A are only partial, but more divergent from the other three strain variants. There are 23 polymorphic sites between strain variants wNdub_A and wNdub2_A, and 42 polymorphic sites between strains wNdub_A and wNcly_A within the 1,887 bp sequenced.

The penetrance of the different strains varies greatly among *Nanos* species and populations (Table 1). The strain variant wNdub_A was common in three of the eight populations of *N. dubitatus* collected across the species range. The strain variant wNvie_A was found in all *N. viettei* and all *N. pseudoviettei* specimens included in this study. The strain variant wNvie2_A was found only in two *N. viettei* specimens collected in Ranomafana. Strikingly, the wNvie2_A-infected specimens are the same *N. viettei* specimens previously described as being the results of introgression events with *N. dubitatus* (Figure 3, Wirta, 2009). Strain variant wNdub2_A was found in one *N. dubitatus* sample collected in Zahamena. We also suggest multiple infections in four *N. dubitatus* samples collected in Zahamena. The sequences from those specimens consistently showed double peaks at several nucleotide positions, similar to double infection with strain variants wNdub_A, and wNdub2_A. Unfortunately, we could not confirm the identity of each variant independently for these multiply infected specimens, so we remain cautious with this particular result. Finally, the strain wNcly_A was described from one unique *N. clypeatus* specimen. The position of the five strain variants described in this study is similar between each phylogenies (Figure S1 and

Figure 1), suggesting lack of recombination between the different *Wolbachia* of the *N. clypeatus* dung beetles clade.

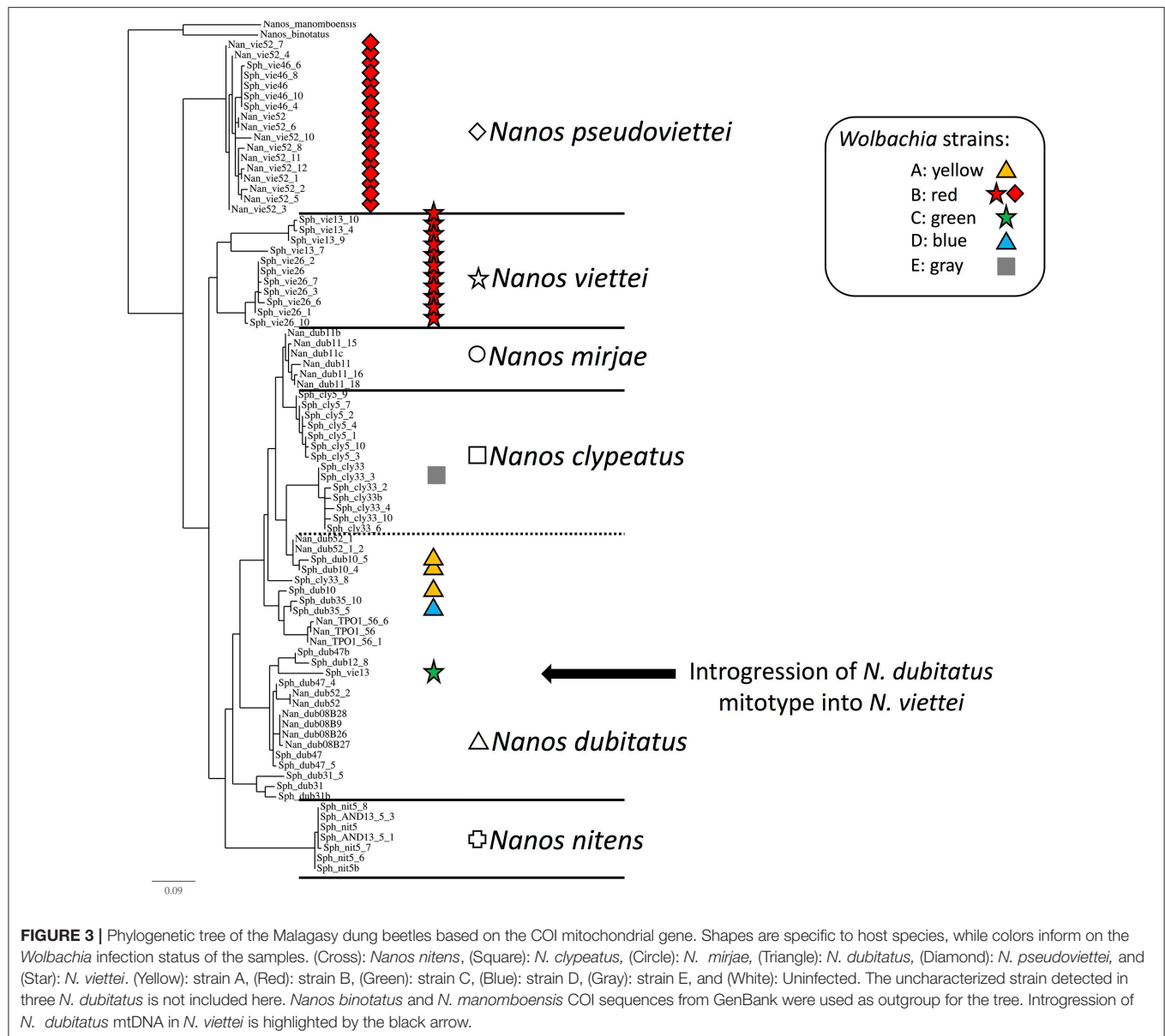
Mitochondrial Diversity

We characterized 67 mitochondrial DNA haplotypes (mitotype) from the 119 beetles included in this study (5 to 17 haplotypes per species). None of the mitotypes characterized from *Wolbachia*-free beetles were found in *Wolbachia*-infected beetles, with the exception of the mitotype associated with *Wolbachia* strain wNcly_A, which is also found in two uninfected individuals. The strain variant wNdub_A is found associated to three of the 17 mitotypes (18%) characterized from *N. dubitatus*. The strain variant wNvie_A is found in association to all 26 mitotypes described from both the *N. viettei* and *N. pseudoviettei* samples ($N = 35$ beetles). The strain variant wNvie2_A is found associated to the unique mitotype characterized from the two *N. viettei* specimens introgressed with *N. dubitatus*. Both strain wNdub2_A and strain wNcly_A were each characterized from one specimen, each carrying their own unique mitotype.

DISCUSSION

Our study is the first to report on *Wolbachia* infections in Malagasy dung beetles. We detected *Wolbachia* infection in 56 specimens (47%) from four of the six dung beetle species screened (67%). This is consistent with current estimates proposing that over 40% of worldwide insect species (Stouthamer et al., 1999), and not so different from lower infection rate estimates detected in other families and species of Coleoptera (Kawasaki et al., 2016; Kajtoch and Kotaskova, 2018; Kolasa et al., 2018). Five of the *Wolbachia* variants we characterized belong to the *Wolbachia* A-supergroup, and are genetically very similar to each other (at most 42 polymorphic sites across 1,800 bp sequenced). The last infection remains to be characterized, or even confirmed. These results suggest the island is potentially not only a biodiversity hotspot for dung beetles, but also a diversity hotspot for the endosymbiotic bacteria they carry.

Each strain shows a very different prevalence within the respective host species. Studies have shown that variations in the prevalence of *Wolbachia* are not rare in insects (Sazama et al., 2019), and these patterns of infection can be the results of the host, the symbiont, or both (Charlat et al., 2005; Hornett et al., 2006). The successful spread of the *Wolbachia* strain wNvie_A in all *N. pseudoviettei* specimens, and almost all *N. viettei* specimens may suggest that the strain is beneficial to its host. As mentioned earlier, CI-inducing strains might rapidly spread in their host populations (Turelli et al., 2018), as the infected females produce viable offspring from mating with either infected or uninfected males, while survival of the offspring from uninfected females is restricted to the females mated to uninfected males (O'Neill et al., 1997; Telschow et al., 2005b). It is thus possible that wNvie_A is a CI-inducing strain. However, *Wolbachia* can also spread across their host populations through enhancing other aspect of its host's fitness. Recent studies have shown that *Wolbachia* can promote the survival of its host against pathogens such as viruses (Hedges et al., 2008; Teixeira et al., 2008; Osborne et al., 2012). Many dung beetles lay their eggs directly in patches of



dung or carrions, or in underground chambers filled with these materials (Viljanen, 2009a,b). The *Nanos* beetles are all rollers, rolling away samples of dung or carrions on which their larvae later feed and develop (Hanski et al., 2008; Rahagalala et al., 2009; Viljanen, 2009b; Viljanen et al., 2010). These environments are often rich in pathogenetic micro-organisms, including bacteria, viruses, and fungi, that may threaten the survival of the offspring. Whether the common strain wNvie_A, or any strain described in this study, can protect their dung beetle hosts against pathogens from their environment remains to be investigated.

In the tropical butterfly *Hypolimnys bolina*, Duploux et al. (2010) have previously shown that geographical variations in the prevalence of a *Wolbachia* strain across the South Pacific populations of the butterfly results from temporal variations in the history of the symbiosis on the different islands. Populations

showing a low infection rate have thus more recently acquired the infection than populations showing a higher infection rate (Duploux et al., 2010). The strain variant wNvie_A is found in almost all *N. viettei* and *N. pseudoviettei* specimens, and in association with a wide diversity of mitotypes in these species, we thus suggest that these host-symbiont associations might be rather old. The other five strains are much rarer in their respective host populations. This pattern might either suggest that those strains are new to their respective hosts and restricted to few mitotypes in the populations, or that the strains are being purge from their host populations, due to potential costs of the symbiont (Charlat et al., 2009; Duploux et al., 2010).

Nanos viettei is a taxonomic unit that is, based on both the mitochondrial and nuclear DNA, very well-separated from all other species from the *N. clypeatus* group (Montreuil et al., 2014).

Wirta (2009) calculated that the split between this species and the other five species in the group occurred at most 7.2 Mya. All strain variants characterized in these dung-beetle species are very similar to each other, but their phylogenetic tree is not congruent with that of their host. Such incongruence has been reported in many host-symbiont associations (Jäckel et al., 2013; Sontowski et al., 2015; Kolasa et al., 2019), including *Wolbachia*-infected insects of Madagascar (Linares et al., 2009; Zouache et al., 2011). Although it is possible that some of our *Nanos* dung-beetle species inherited their *Wolbachia* infections from a common ancestor, while others have lost or gained new infections during species radiation, our data also does not suggest radiation of *Wolbachia* in parallel to the radiation of *N. clypeatus* group.

Although mostly vertically transmitted from the mother to the offspring, *Wolbachia* is also known to transfer horizontally between host species (Vavre et al., 1999; Bailly-Bechet et al., 2017). Such transfer of infection may occur during hybridization events between species. For example, in *Drosophila mauritiana*, different populations bear the mitotypes of another fruitfly species, *D. simulans*, and variants of the *Wolbachia* strain wMau, most likely due to transfer of both cytoplasmic entities during recent introgression events (Rousset and Solignac, 1995). However, other ecological links are likely to also support the horizontal transfer of symbiotic bacteria between host species. Species with similar resources, such as larval host plant, or species sharing parasites, such as parasitoid or mite infections, may thus also share similar *Wolbachia* infections (Stahlhut et al., 2010). Ecological studies on the *Nanos* species have shown that *N. viettei* mate with other *Nanos* species in the laboratory (Viljanen, 2009a,b). The survival of the offspring has however never been investigated. Furthermore, previous phylogenetic studies do not suggest introgression between the populations of *N. pseudoviettei* and *N. viettei* (Miraldo and Hanski, 2014). In contrast, the two species co-occur in the northern limit of *N. viettei* geographical range (Viljanen, 2009a; Miraldo et al., 2011; Montreuil et al., 2014). There, these two species, which are generalists in their choice of dung, may enter in contact and share dung patches or parasites. These ecological links may have facilitated the horizontal transfer of *Wolbachia* between the two species (Kolasa et al., 2019). Unfortunately, little is known about the parasitoid community associated to the Malagasy dung beetles, and no study has ever investigated the potential for dung as a suitable environment for symbiont transfer between carrion insects.

Strikingly, some of the rare strain variants characterized in this study are found in association with specimens showing clear genetic patterns of introgression. Strain variant wNvie2_A, a close variant of wNvie_A, is found in two *N. viettei* that carry the mitochondrial DNA from *N. dubitatus*. These particular specimens were collected from a region where extensive hybridization is known to happen between the two sympatric host species (Viljanen, 2009a; Wirta, 2009). Previous studies described these introgressed individuals as clear units within the *N. viettei* species that were however separated by unknown processes (Miraldo and Hanski, 2014). We suggest that sympatry of the two species may have facilitated the transfer of the *Wolbachia* between specimens. However, the divergence between strain variants

wNvie_A and wNvie2_A may have led these specimens to become incompatible potentially due to CI. Such postzygotic incompatibility would have, with time, led to radiation of the introgressed lineage carrying wNvie2_A from the other *N. viettei*, wNvie_A-infected lineages. Simultaneously, because wNvie2_A-infected *N. viettei* individuals are morphologically similar to *N. viettei*, they might rarely mate with the locally occurring *N. dubitatus* specimens. Again, with time, prezygotic isolation may have also isolated this lineage from the other *N. dubitatus* lineages. The strain wNdub_A, a second close variant of wNvie_A, is found in *N. dubitatus* specimens. Both strains and their respective host species do not occur in sympatry in Madagascar. Although wNdub_A was identified in association to a rather wide diversity of mitotypes in *N. dubitatus*, our current sample size is unfortunately too small to further speculate on the potential phenotypic effects this strain might have on its hosts, or on the origin of the strain in *N. dubitatus*.

Despite the fact that our restricted sample size is preventing a more comprehensive assessment of prevalence rates and any evidence of geographical variation between collection locations, our results still support the idea that *Wolbachia* might play a key role in the ecology and evolutionary biology of the endemic Malagasy dung beetles. Viljanen (2009b) showed that some *Nanos* species can mate in the laboratory, thus it is possible for future studies to confirm whether the different strains of *Wolbachia* induce CI in these insects. Only then will we be able to confirm whether *Wolbachia* play a key role in designing host species boundaries, or can in any ways enhance their host fitness and contribute to niche colonization and rapid geographical expansion of these ecological successful dung beetle species.

DATA AVAILABILITY

The datasets generated for this study can be found in GenBank, Accession codes are provided in the manuscript.

ETHICS STATEMENT

The study only include work on DNA extracts from samples collected under the Academy of Finland projects to late professor Ilkka Hanski. All samples were collected prior to 2014, no Nagoya protocol was then required.

AUTHOR CONTRIBUTIONS

AD designed the research and wrote the manuscript. AM and AD collected the data, analyzed the data, and revised previous versions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2019.00157/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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